Heterozygous HIF-1α deficiency impairs carotid body-mediated systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia

Ying-Jie Peng[1], Guoxiang Yuan[1], Deviprasadh Ramakrishnan[2], Suresh D. Sharma[2], Marta Bosch-Marce[3], Ganesh K. Kumar[2], Gregg L. Semenza[3] and Nanduri R. Prabhakar[1]

Departments of [1]Physiology & Biophysics and [2]Biochemistry, Case Western Reserve University, Cleveland, OH, 44106, USA
[3]Vascular Biology Program, Institute for Cell Engineering; Departments of Pediatrics, Medicine, Oncology, Radiation Oncology; and McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Chronic intermittent hypoxia (CIH) occurs in patients with sleep apnoea and has adverse effects on multiple physiological functions. Previous studies have shown that reflexes arising from carotid bodies mediate CIH-evoked cardio-respiratory responses, and reactive oxygen species (ROS) play important roles in eliciting systemic responses to CIH. Very little is known about the molecular mechanisms underlying CIH. The transcriptional activator hypoxia-inducible factor-1 (HIF-1) mediates a broad range of cellular and systemic responses to hypoxia, and HIF-1 is activated in cell cultures exposed to IH. In the present study we examined whether CIH activates HIF-1 and if so whether it contributes to cardio-respiratory responses and ROS generation in mice. Experiments were performed on male littermate wild-type (WT) and heterozygous (HET) mice partially deficient in HIF-1α, the O_2 regulated subunit of the HIF-1 complex. Both groups of mice were exposed to either 10 days of CIH (15 s of hypoxia followed by 5 min of normoxia, 9 episodes h^{-1}, 8 h day^{-1}) or to 10 days of 21% O_2 (controls). Carotid body response to hypoxia was augmented, and acute intermittent hypoxia (AIH) induced sensory long-term facilitation (sLTF) of the chemoreceptor activity in CIH-exposed WT mice. In striking contrast, hypoxic sensory response was unaffected and AIH was ineffective in eliciting sLTF in CIH-exposed HET mice. Analysis of cardio-respiratory responses in CIH-exposed WT mice revealed augmented hypoxic ventilatory response, LTF of breathing, elevated blood pressures and increased plasma noradrenaline. In striking contrast these responses were either absent or attenuated in HET mice exposed to CIH. In CIH-exposed WT mice, ROS were elevated and this response was absent in HET mice. Manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride, a potent scavenger of superoxide, not only prevented CIH-induced increases in ROS but also CIH-evoked HIF-1α up-regulation in WT mice. These results indicate that: (a) HIF-1 activation is critical for eliciting CIH-induced carotid body-mediated cardio-respiratory responses; (b) CIH increases ROS; and (c) the effects of CIH involve complex positive interactions between HIF-1 and ROS.

(Received 23 May 2005; accepted after revision 8 September 2006; first published online 14 September 2006)
Corresponding author N. R. Prabhakar: Department of Physiology & Biophysics, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44109, USA. Email: nrp@case.edu

Patients with recurrent apnoeas (transient cessation of breathing) as a consequence of sleep-disordered breathing experience chronic intermittent hypoxia (CIH). Carotid bodies are the sensory organs for detecting the changes in arterial blood oxygen. Recent studies have shown that CIH selectively augments carotid body sensory response to hypoxia (Peng & Prabhakar, 2004; Rey *et al.* 2004) and acute intermittent hypoxia (AIH) leads to sensory long-term facilitation (sLTF) in CIH but not in control carotid bodies (Peng *et al.* 2003). The effects are selective to CIH, because comparable durations of continuous hypoxia neither increased the hypoxic sensory response nor induced sLTF (Peng *et al.* 2003; Peng & Prabhakar, 2004). Studies on recurrent apnoea patients and in experimental animals exposed to CIH suggest that reflexes arising from the carotid bodies are critical

 DOI: 10.1113/jphysiol.2006.114033

for eliciting CIH-induced elevated blood pressures, augmented sympathetic activity, and abnormalities in the neurochemical control of breathing (Fletcher *et al.* 1992; Cistulli & Sullivan, 1994; Kara *et al.* 2003). However, very little is known about the molecular mechanisms underlying CIH.

The transcriptional activator hypoxia-inducible factor 1 (HIF-1) is a global regulator of O_2 homeostasis that controls multiple physiological processes and regulates the expression of hundreds of genes (Manalo *et al.* 2005; Wenger *et al.* 2005; Hirota & Semenza, 2006). HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit (Wang *et al.* 1995). Complete HIF-1α deficiency results in embryonic lethality at mid-gestation, whereas *Hif1a*$^{+/-}$ heterozygous (HET) mice, which are partially deficient in HIF-1α expression, develop normally and are indistinguishable from wild-type (WT) littermates under normoxic conditions (Iyer *et al.* 1998; Yu *et al.* 1999). However, the carotid body response to hypoxia is selectively impaired in adult HET mice, suggesting that HIF-1 plays an essential role in O_2 sensing by the carotid body (Kline *et al.* 2002). Recently, we reported that IH increases HIF-1α expression and HIF-1-mediated transcription via a novel calcium/calmodulin–protein kinase-dependent mechanism in pheochromocytoma (PC12) cells (Yuan *et al.* 2005). Whether HIF-1α expression increases in CIH-exposed animals and, if so, whether HIF-1 contributes to CIH-evoked cardio-respiratory responses, however, have not been examined. Therefore, the first objective of the present study was to examine the effects of CIH on HIF-1α expression as well as carotid body and peripheral chemoreceptor-mediated cardio-respiratory responses in adult WT and HET mice.

Recent studies suggest that CIH increases reactive oxygen species (ROS), as shown by decreased aconitase activity (Peng *et al.* 2003; Yuan *et al.* 2004; Kumar *et al.* 2006), and increased protein oxidation, measured as thiobarbituric acid reactive substances (TBARS; Ramanathan *et al.* 2005). Increased generation of ROS was also reported in humans experiencing CIH as a consequence of recurrent apnoeas (Dyugovskaya *et al.* 2002). In experimental models, antioxidants prevent CIH-induced changes in the carotid body activity (Peng *et al.* 2003), enhanced transmitter secretion (Kim *et al.* 2004), increased blood pressure (Kumar *et al.* 2006), and immediate early gene activation (Yuan *et al.* 2004). These studies implicate increased ROS as an important signal in eliciting systemic and cellular responses to CIH. The second objective of the present study was to examine whether CIH increases ROS in mice and, if so, whether HIF-1 contributes to this response.

Methods

General preparation of animals

Experiments were approved by the Institutional Animal Care and Use Committee of the Case Western Reserve University and were performed on male, age-matched (10–15 weeks old) WT and HET mice (Iyer *et al.* 1998; Yu *et al.* 1999; Kline *et al.* 2002) by individuals blinded to the genotype. The average weights of the HET and WT mice were 29 ± 0.6 and 28 ± 1.1 g, respectively ($P > 0.05$, ANOVA). Experiments were performed on awake, unrestrained as well as anaesthetized mice.

In the experiments requiring sedation, mice were anaesthetized with intraperitoneal injections of urethane (1.2 g kg^{-1}, Sigma). The choice of urethane was based on the report that acid–base status of arterial blood is well maintained in experimental animals (Buelke-Sam *et al.* 1978). Supplemental doses, 10% of the initial dose, of anaesthetic were given when corneal reflexes and responses to toe pinch persisted. In anaesthetized animals, routine surgical procedures included tracheal intubation and catheterization of a femoral artery and vein. Blood samples were collected via the arterial catheter for blood gas analysis. Systemic administration of fluids, when necessary was accomplished via the venous catheter. Mice were allowed to breathe spontaneously. Core body temperature was monitored by a rectal thermistor probe and maintained at $37 \pm 1^{\circ}$C by a heating pad. At the end of the experiment, mice were killed by intracardiac injection (0.1 ml) of euthanasia solution (Beuthanasia-D Special, Schering-Plough, Kenilworth, NJ, USA).

Chronic exposure to intermittent hypoxia (CIH)

Unrestrained, freely moving mice housed in feeding cages were exposed to a CIH protocol consisting of 15 s of 5% inspired O_2 followed by 5 min of room air (normoxia), 9 episodes h^{-1} and 8 h day^{-1} for 10 days as previously described (Peng & Prabhakar, 2004). Briefly, mice were placed in a specialized chamber, which was flushed with alternating cycles of pure nitrogen and compressed air. During hypoxia, inspired O_2 levels reached 5% O_2 (nadir) within 50 ± 2 s and was maintained at this level for 15 s. This was followed by room air which reached 21% O_2 within 55 ± 4 s and was maintained for 5 min. The gas flows were regulated by timer-controlled solenoid valves. Ambient oxygen levels in the chamber were continuously monitored by an O_2 analyser (Beckman; Model OM-11). Inspired CO_2 in the chamber was monitored continuously by an infrared analyser and maintained between 0.2 and 0.5% (Beckman; Model LB-2). Control experiments were performed on mice exposed to alternating cycles of compressed room air instead of hypoxia in the same chamber. Measurements of ventilation, blood pressure,

carotid body activity and plasma noradrenaline (NA) were made 3 h following termination of 10 days of CIH or normoxia except where otherwise noted.

Recording of carotid body sensory activity

Sensory activity from carotid bodies *ex vivo* was recorded as previously described (Peng *et al.* 2003). Briefly, carotid bodies along with the sinus nerves were harvested from anaesthetized mice, placed in a recording chamber (volume, 250 μl) and superfused with warm physiological saline (35°C) at a rate of 2 ml min^{-1}. The composition of the medium was (mM): NaCl (125), KCl (5), $CaCl_2$ (1.8), $MgSO_4$ (2), NaH_2PO_4 (1.2), $NaHCO_3$ (25), D-glucose (10), sucrose (5), and the solution was bubbled with 95% O_2–5% CO_2. To facilitate recording of clearly identifiable action potentials, the sinus nerve was treated with 0.1% collagenase for 5 min. Action potentials (2–5 active units) were recorded from one of the nerve bundles with a suction electrode and stored in a computer via an A/D translation board (PowerLab/8P, AD Instruments Pty Ltd, Australia). The criteria for chemoreceptor activity include increased sensory activity in response to stagnant hypoxia (i.e. interrupting the superfusion for 5 min) and return to baseline after resuming the superfusion. 'Single' units were selected based on the height and duration of the individual action potentials using a spike discrimination program (Spike Histogram Program, Power Laboratory, AD Instruments). In each carotid body, at least two chemoreceptor units were analysed. The P_{O_2} and P_{CO_2} of the superfusion medium were determined by a blood gas analyser (ABL 5, Radiometer, Copenhagen, Denmark).

Measurements of ventilation, metabolic variables and arterial blood pressure (BP)

In unanaesthetized animals, ventilation was monitored using whole body plethysmograph as previously described (Kline *et al.* 2002). Briefly, animals were placed in a 600-ml Lucite chamber containing an inlet port for gas administration and were allowed to acclimate for 1 h in room air. The chamber was connected to a high-gain differential pressure transducer (Valydine MP45, Validyne, North Ridge, CA, USA). As the animal breathed, changes in pressure were converted to signals representing tidal volume (V_T), which were amplified (BMA 830; CWE, Ardmore, PA, USA), recorded and stored in a computer via an A/D translation board (PowerLab/8P) for further analysis. Oxygen consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) were determined by the open-circuit method as described by Frappell *et al.* (1992). The following equations were used to calculate $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$:

$$\dot{V}_{O_2} = \dot{V}_E(F_{iO_2} - F_{eO_2})/(1 - F_{iO_2})$$

and

$$\dot{V}_{CO_2} = \dot{V}_E(F_{eCO_2} - F_{iCO_2})/(1 - F_{iCO_2})$$

wherein i denote ingoing gas, and e denotes outgoing gas, $\dot{V}$ the flow and F fractional concentration (Frappell *et al.* 1992). Sighs, sniffs and movement-induced changes in breathing and metabolic variables were monitored and excluded in the analysis. All recordings were made at an ambient temperature of 25 ± 1°C.

BP was monitored by the tail cuff method in unanaesthetized mice using a non-invasive BP system (AD Instruments). Mice were placed in the restrainer provided by the manufacturer. Mice were allowed to acclimate for at least 1 h prior to BP measurements.

In anaesthetized animals, integrated efferent phrenic nerve activity was monitored as an index of central respiratory neuronal output. The phrenic nerve was isolated unilaterally at the level of the C3 and C4 spinal segments. The nerve was cut distally, and placed on bipolar stainless steel electrodes. Unilateral sectioning of a small branch of phrenic nerve had no discernable effect on spontaneous breathing. The electrical activity was filtered (band pass 0.3–1.0 kHz), amplified, and passed through Paynter filters (time constant of 100 ms; CWE Inc.) to obtain a moving average signal. Data were stored in the computer for further analysis.

Measurements of plasma noradrenaline (NA)

Blood samples (~300 μl) were collected from anaesthetized mice by cardiac puncture, and placed in heparinized (30 U ml^{-1} of blood) ice-cold micro centrifuge tubes. Plasma was separated by centrifugation and stored at −80°C. Plasma NA was determined by high pressure liquid chromatography combined with electrochemical detection (HPLC-ECD) using dihydroxybenzylamine as an internal standard (Kim *et al.* 2004). The NA levels were corrected for recovery loss and expressed as nanograms of NA (100 ml of plasma)$^{-1}$.

Analysis of HIF-1α and HIF-1β protein

Brain tissues (cortical region) were removed from anaesthetized mice, frozen in liquid nitrogen and stored at −80°C. HIF-1α and HIF-1β proteins were analysed by immunoblot assay (Bergeron *et al.* 2000). The following antibodies were used: anti-HIF-1α monoclonal antibody H1α67 (Zhong *et al.* 2000) at 1 : 500 dilution and HIF-1β monoclonal antibody H1β234 (Zagzag *et al.* 1999) at 1 : 1500 dilution. Antibody complexes on the membrane were visualized with enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Measurements of thio-barbituric acid reactive substances (TBARS)

Cortical tissue was homogenized in 10 volumes of 20 mM phosphate buffer (pH 7.4) at 4°C and the resulting homogenate was centrifuged at 500 *g* for 10 min at 4°C. TBARS were analysed in the supernatant as previously described (Ramanathan *et al.* 2005). Briefly, 100 μl of either sample or the standard was added to 50 μl of 8.1% (w/v) SDS, 375 μl of 20% (v/v) acetic acid and 375 μl of 0.8% (w/v) thiobarbituric acid. The samples were heated for 60 min in a boiling water bath followed by incubation on an ice bath for 10 min and centrifuged at 3000 *g* for 15 min. The supernatant was removed and the absorbance of the solution was monitored at 532 nm. Malondialdehyde (MDA) was used as a standard, and the level of TBARS was reported in nanomoles of MDA formed per milligram of protein.

Experimental protocols

Series 1. The effects of brief hyperoxia (100% O_2; Dejour's test) on efferent phrenic nerve activity was assessed in anaesthetized mice exposed to CIH or normoxia for 10 days (WT; $n = 5$ mice for each condition; HET; $n = 6$ normoxia, $n = 7$ for CIH). Baseline phrenic activity was recorded while animals breathed 21% O_2 for 5 min followed by 100% O_2 for 20 s. Phrenic nerve activity was analysed for 1 min during 21% O_2 immediately prior to 100% O_2 exposure and during the last 15 s of hyperoxia (the initial 5 s was excluded because of the dead space in the tracheal catheter). At the end of the experiments arterial blood samples were collected for blood gas analysis. In another group of experiments, carotid body responses to graded hypoxia, sodium cyanide (NaCN, 3 μg ml^{-1}) and sLTF were determined. Experiments were performed on 12 carotid bodies from 6 WT mice and 14 carotid bodies from 7 HET mice exposed to normoxia; and 14 carotid bodies from 7 WT mice and 12 carotid bodies from 6 HET mice exposed to CIH.

Series 2. Arterial BP was determined in unanaesthetized HET and WT mice ($n = 8$ each) before and after CIH such that each animal served as its own control. Basal BP was recorded while the mice breathed room air. Following the measurements of BP, hypoxic and hypercapnic ventilatory responses were determined in the same mice. For measuring hypoxic ventilatory response (HVR), mice were exposed to 100, 21, and 12% O_2–balance N_2. Each gas challenge was given for 5 min. The protocols were repeated after a 20 min interval. O_2 consumption and CO_2 production were measured at the end of each 5 min challenge. For measuring hypercapnic ventilatory response, mice inspired 100% O_2 for 5 min followed by 5% CO_2–balance O_2. The protocol was repeated twice, with a 20 min interval between each protocol.

Series 3. Long-term facilitation (LTF) of breathing was analysed in unanaesthetized WT and HET mice ($n = 8$ each) as described in Results.

Series 4. Changes in plasma NA were analysed in anaesthetized WT and HET mice exposed to CIH or normoxia ($n = 7$ each).

Series 5. HIF-1α and HIF-1β protein levels were analysed by immunoblot assay in cortical tissue collected from anaesthetized WT and HET mice exposed to CIH or normoxia ($n = 5$ mice in each group).

TBARS were analysed in the same tissue samples. In another set of experiments, analysis of TBARS and HIF-1α were performed in CIH and normoxic mice ($n = 5$ each) treated for 10 days with the superoxide dismutase mimetic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP, Alexis Chemicals, 5 mg kg^{-1} day^{-1} I.P.), a potent scavenger of $O_2^{\bullet -}$.

Data analysis

The following variables were analysed in unanaesthetized mice: tidal volume (V_T; μl); respiratory rate (RR min^{-1}); minute ventilation ($\dot{V}_E$; ml min^{-1} (g body wt)$^{-1}$); O_2 consumption ($\dot{V}_{O_2}$; ml min^{-1}); CO_2 production ($\dot{V}_{CO_2}$; ml min^{-1}); systolic, diastolic and mean arterial BP (mmHg). Respiratory variables (RR and V_T) were averaged for 15 consecutive breaths over 5 min of inspired O_2 and CO_2 challenges. V_T and $\dot{V}_E$ were normalized to the body weight of the animals. Each data point represents the average of two trials in a given animal for a given gas challenge. In anaesthetized animals, the following respiratory variables were analysed: respiratory rate (RR; phrenic bursts min^{-1}), amplitude of the integrated phrenic nerve activity (a.u., arbitrary units) and minute neural respiration (number of phrenic bursts min^{-1}, RR × amplitude of the integrated phrenic nerve activity, a.u.). Carotid body sensory activity (discharge from 'single' units) was averaged during 3 min of baseline and during the 3 min of gas challenge and expressed as impulses s^{-1} unless otherwise stated. All data are presented as mean ± S.E.M. Statistical significance was assessed by either two-way ANOVA with repeated measures followed by Tukey's test. P values < 0.05 were considered significant.

Results

Effects of CIH on carotid body activity

Ventilatory response to acute hyperoxia (Dejour's test). The magnitude of the transient ventilatory decline in response to a brief hyperoxic exposure was used as an index of peripheral chemoreceptor, especially the carotid body, sensitivity (Dejour, 1962). CIH-exposed WT mice

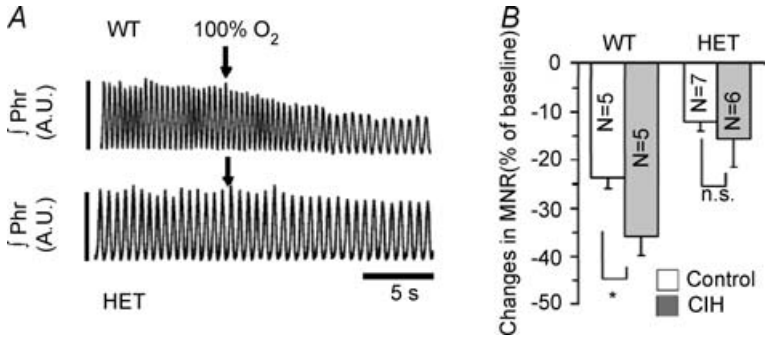

Figure 1. Effect of brief hyperoxia (Dejour's test) on minute neural respiration (MNR)
A, efferent phrenic nerve responses to brief (20 s) exposure to 100% O_2 (at arrow) in anaesthetized, spontaneously breathing *Hif1a*$^{+/+}$ (WT, top panel) and *Hif1a*$^{+/-}$ (HET, bottom panel) mice exposed to CIH for 10 days. ∫Phr, integrated efferent phrenic nerve activity. A.U., arbitrary units. *B*, mean (± S.E.M.) change in MNR [$(MNR_{21\%} - MNR_{100\%})/MNR_{21\%}$]. *N*, number of mice. $^*P < 0.05$; N.S., not significant.

manifested a significantly greater depression of minute neural respiration than HET mice exposed to CIH (Fig. 1). These observations indicated that CIH augments peripheral chemoreceptor sensitivity in WT but not in HET mice.

Carotid body sensory response to graded hypoxia. To directly establish the effects of CIH on chemoreceptor responses to hypoxia, sensory discharge was recorded from *ex vivo* carotid bodies. Examples of carotid body sensory response to hypoxia ('single' fibre response) from WT and HET mice and plots of mean data are presented in Fig. 2. Carotid bodies from CIH-exposed WT mice manifested a greater magnitude of hypoxic sensory response than the controls (i.e. carotid bodies from normoxic WT mice). Control HET mice exhibited a blunted hypoxic sensory response and CIH had little effect in augmenting the hypoxic sensitivity (Fig. 2*B*). Carotid bodies from both groups of mice, however, responded to cyanide ($3\,\mu g\,ml^{-1}$) with comparable increase in sensory activity before and after CIH (before CIH: WT, $+8.1 \pm 1$ impulses s^{-1} and HET, $+8.6 \pm 0.9$ impulses s^{-1}; after IH: WT, $+9.6 \pm 2.3$ impulses s^{-1} and HET, $+8.7 \pm 1.7$ impulses s^{-1}).

Sensory long-term facilitation (sLTF) of the carotid body. Previous study has shown that acute intermittent hypoxia (AIH) leads to sLTF of the carotid body only after CIH (Peng *et al.* 2003). We examined whether HIF-1 plays a role in eliciting sLTF of the carotid body. AIH was ineffective in inducing sLTF in control WT and HET mice. However, sLTF could readily be elicited by AIH in WT but not in HET mice exposed to CIH (Fig. 3).

Ventilatory and blood pressure responses to CIH

Hypoxic ventilatory response (HVR). Examples of HVR and mean data in both groups of mice as

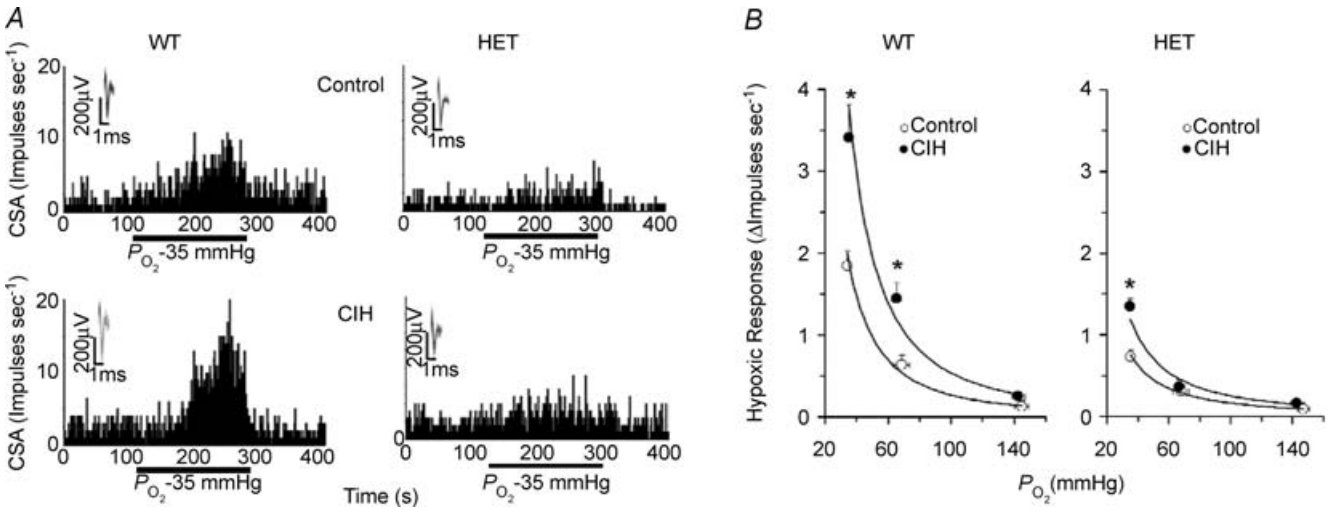

Figure 2. Effect of CIH on carotid body sensory activity
A, sensory responses to hypoxia (black bars) in *Hif1a*$^{+/+}$ (WT, left panel) and *Hif1a*$^{+/-}$ (HET, right panel) mice exposed either to normoxia (control) or to CIH for 10 days. P_{O_2}, partial pressure of O_2 in the perfusate. Superimposed action potential of 'single' fibre from which the data were derived is shown (inset). *B*, mean sensory response to graded hypoxia presented as change in impulses s^{-1} (hypoxia – baseline). Data presented are mean ± S.E.M. $^*P < 0.05$. The numbers of animals in each group are given in Methods.

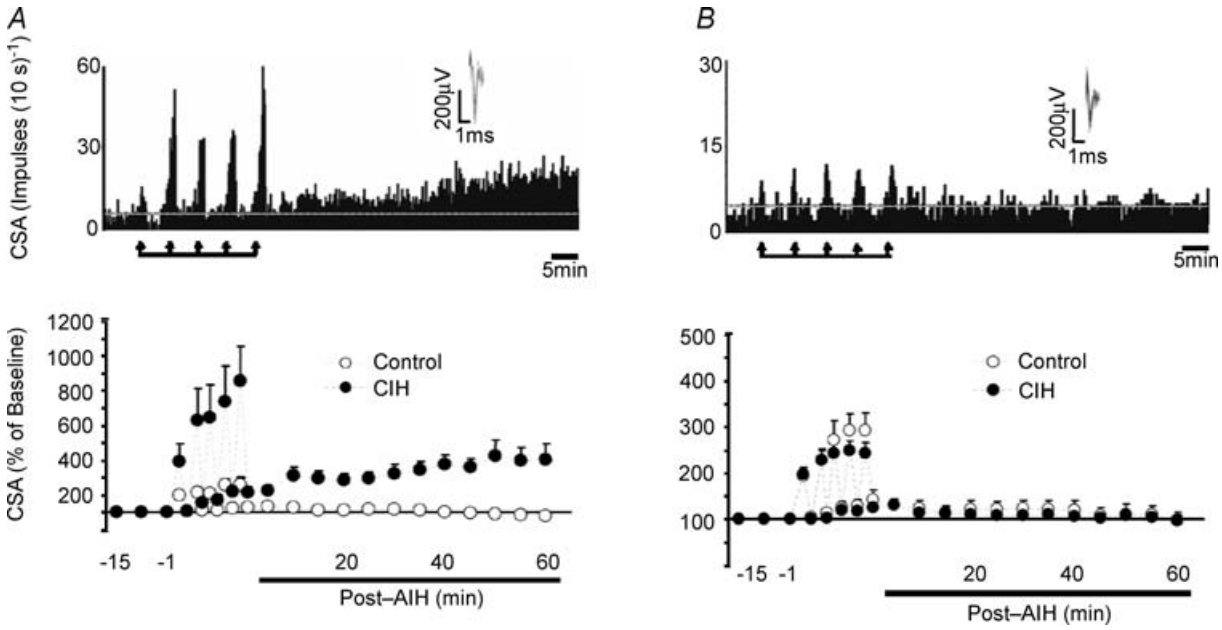

Figure 3. Sensory long-term facilitation (sLTF) of the carotid body
Top panels, sLTF of the carotid body from *Hif1a*$^{+/+}$ (WT, left panel) and *Hif1a*$^{+/-}$ (HET, right panel) mice exposed to CIH for 10 days. Sensory LTF was induced by five brief (30 s) episodes of acute hypoxia (at arrows). Continuous white line indicates the baseline sensory activity. Superimposed action potential of a 'single' fibre from which the data were derived is shown (inset). Bottom panels, mean (+ S.E.M.) sensory activity presented as percentage of baseline activity. Post-AIH, post acute intermittent hypoxia. The number of animals in each group is given in Methods.

percentage controls are presented in Fig. 4. Absolute values of respiratory changes and metabolic variables ($\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, $\dot{V}_E/\dot{V}_{O_2}$, a measure of convective requirement) are given in Table 1. Prior to CIH, ventilation increased in both groups of mice in response to 21 and 12% O_2 and the magnitude of increases were comparable. $\dot{V}_E/\dot{V}_{O_2}$ during 21 and 12% O_2 tended to be higher in HET than WT, but the differences were not statistically significant ($P > 0.05$).

After CIH, WT mice responded with greater increases in $\dot{V}_E$ during 21 and 12% O_2 than pre-CIH. This increase in $\dot{V}_E$ was primarily due to significant increases in RR (Fig. 4 and Table 1). However, the ratio of $\dot{V}_E/\dot{V}_{O_2}$ during 21% O_2 was unchanged, whereas it significantly increased during 12% O_2 ($P < 0.05$). In contrast, ventilatory responses (RR, V_T, $\dot{V}_E$) and $\dot{V}_E/\dot{V}_{O_2}$ during 21 and 12% O_2 remained nearly the same in CIH exposed HET mice compared with pre-CIH

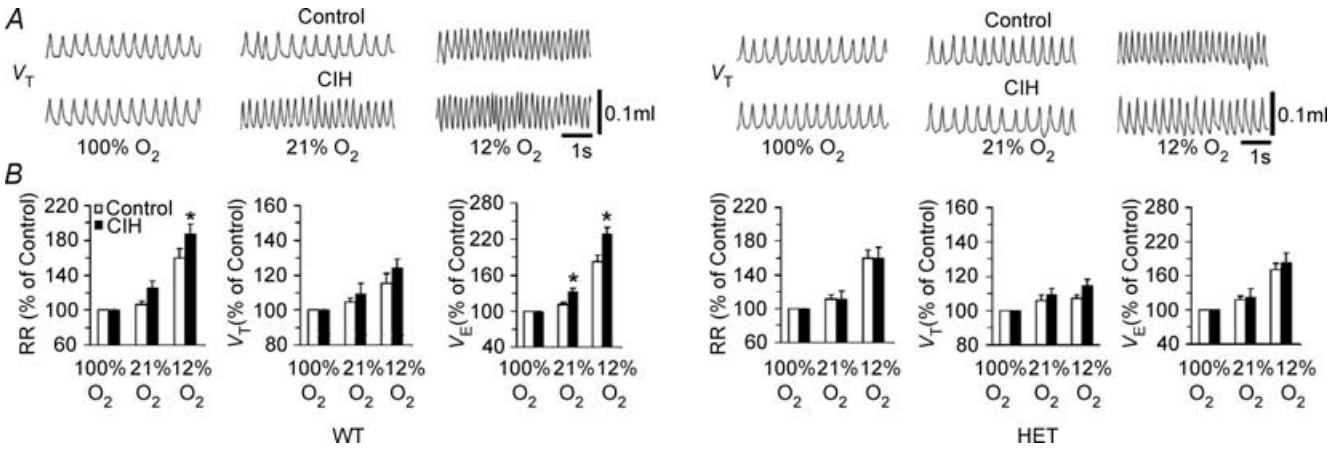

Figure 4. Effect of CIH on hypoxic ventilatory responses
A, ventilation while breathing 100, 21 and 12% O_2 in *Hif1a*$^{+/+}$ (WT, left panel) and *Hif1a*$^{+/-}$ (HET, right panel) mice before (control) and after 10 days of CIH. V_T, tidal volume. *B,* mean respiratory rate (RR), V_T, and minute ventilation ($\dot{V}_E$) presented as percentage of baseline activity (100% O_2). Open and closed bars represent responses before after CIH, respectively. Data presented are mean + S.E.M. from eight animals in each group. *$P < 0.05$.

Table 1. Hypoxic ventilatory response before and after CIH in WT and HET mice

	Before CIH			After CIH		
	100% O_2	21% O_2	12% O_2	100% O_2	21% O_2	12% O_2
WT ($n = 8$)						
RR (breaths min^{-1})	167 ± 7	176 ± 4	265 ± 12	153 ± 6	189 ± 7	284 ± 13*
V_T ($\mu l\ g^{-1}$)	1.64 ± 0.15	1.71 ± 0.14	1.86 ± 0.14	1.82 ± 0.17	1.94 ± 0.14	2.22 ± 0.17
$\dot{V}_E$ (ml g^{-1} min^{-1})	0.27 ± 0.02	0.30 ± 0.02	0.49 ± 0.04	0.28 ± 0.02	0.37 ± 0.03*	0.64 ± 0.06*
$\dot{V}_{O_2}$ (ml g^{-1} min^{-1})	0.09 ± 0.01	0.07 ± 0.005	0.04 ± 0.004	0.11 ± 0.01	0.08 ± 0.008	0.04 ± 0.004
$\dot{V}_{CO_2}$ (ml g^{-1} min^{-1})	0.04 ± 0.004	0.05 ± 0.005	0.03 ± 0.002	0.04 ± 0.003	0.05 ± 0.003	0.03 ± 0.002
$\dot{V}_E/\dot{V}_{O_2}$	3.22 ± 0.30	4.63 ± 0.45	14.7 ± 2.13	2.87 ± 0.36	4.85 ± 0.59	17.7 ± 1.73*
HET ($n = 8$)						
RR (breaths min^{-1})	156 ± 7	174 ± 5	253 ± 14	157 ± 12	169 ± 9	249 ± 14
V_T ($\mu l\ g^{-1}$)	1.86 ± 0.13	1.99 ± 0.12	2.00 ± 0.14	1.93 ± 0.13	2.08 ± 0.09	2.18 ± 0.15
$\dot{V}_E$ (ml g^{-1} min^{-1})	0.28 ± 0.01	0.34 ± 0.02	0.50 ± 0.03	0.30 ± 0.03	0.35 ± 0.02	0.53 ± 0.03
$\dot{V}_{O_2}$ (ml g^{-1} min^{-1})	0.10 ± 0.008	0.06 ± 0.004	0.03 ± 0.002	0.09 ± 0.005	0.06 ± 0.005	0.03 ± 0.004
$\dot{V}_{CO_2}$ (ml g^{-1} min^{-1})	0.05 ± 0.006	0.04 ± 0.002	0.03 ± 0.003	0.05 ± 0.006	0.05 ± 0.004	0.03 ± 0.003
$\dot{V}_E/\dot{V}_{O_2}$	3.09 ± 0.32	5.63 ± 0.38	15.63 ± 1.63	3.48 ± 0.27	6.11 ± 0.36	16.5 ± 1.24

Data presented are mean + S.E.M. n = number of mice. *$P < 0.05$ compared to the responses before CIH.

controls (Fig. 4 and Table 1). Changes in $\dot{V}_{CO_2}$ (Table 1) and arterial blood gases (Table 2) were comparable in HET and WT mice before and after CIH.

Hypercapnic ventilatory response. Before CIH, both groups of mice responded to 5% inspired CO_2 with increases in RR, V_T and $\dot{V}_E$ (Fig. 5). Unlike the HVR, following CIH both WT and HET mice responded with augmented ventilatory responses to CO_2 (Table 3).

Table 2. Changes in arterial blood gases in WT and HET mice before and after CIH

	WT		HET	
	Before CIH ($n = 5$)	After CIH ($n = 5$)	Before CIH ($n = 5$)	After CIH ($n = 4$)
P_{aO_2}	129 ± 5	134 ± 4	133 ± 6	135 ± 3
P_{aCO_2}	36 ± 3	37 ± 3	37 ± 3	38 ± 3
pH	7.27 ± 0.02	7.26 ± 0.05	7.28 ± 0.02	7.27 ± 0.01

Data presented are mean + S.E.M. n = number of mice.

Long-term facilitation (LTF) of breathing. The following strategy was employed to examine the effect of CIH on LTF of breathing in unanaesthetized mice. If LTF of breathing is induced by CIH, then baseline breathing should be elevated immediately after terminating CIH, and should return to control levels within a few hours after placing mice in room air. To test this possibility, baseline ventilation was monitored in WT and HET

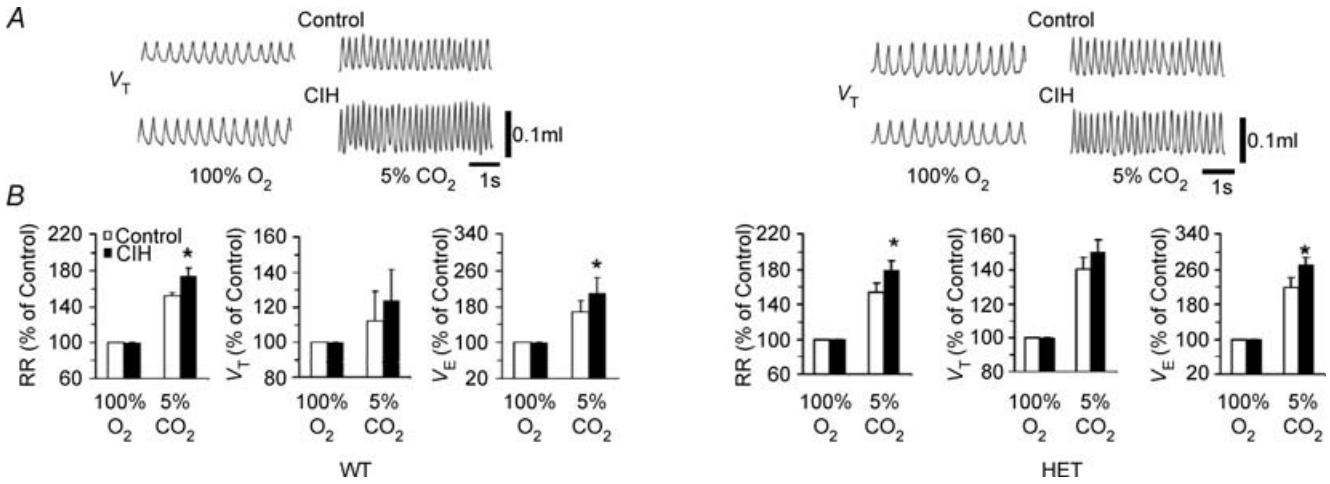

Figure 5. Effect of CIH on hypercapnic ventilatory responses
A, ventilation while breathing 100% O_2 and 5% CO_2 in *Hif1a*$^{+/+}$ (WT, left panel) and *Hif1a*$^{+/-}$ (HET, right panel) mice before (control) and after 10 days of CIH. V_T, tidal volume. *B,* mean respiratory rate (RR), tidal volume (V_T), and minute ventilation ($\dot{V}_E$) presented as percentage of baseline activity (100% O_2). Open and filled bars represent responses before after CIH, respectively. Data presented are mean + S.E.M. from eight animals in each group. *$P < 0.05$.

Table 3. Ventilatory responses to hyperoxic hypercapnia in WT and HET mice before and after CIH

	Before CIH		After CIH	
	100% O_2	5% CO_2	100% O_2	5% CO_2
WT ($n = 8$)				
RR (breaths min^{-1})	167 ± 7	254 ± 9	153 ± 6	265 ± 14
V_T (μl g^{-1})	1.64 ± 0.15	2.09 ± 0.19	1.82 ± 0.17	2.57 ± 0.23
$\dot{V}_E$ (ml g^{-1} min^{-1})	0.27 ± 0.02	0.52 ± 0.03	0.28 ± 0.02	0.67 ± 0.05*
HET ($n = 8$)				
RR (breaths min^{-1})	156 ± 7	238 ± 15	156 ± 12	274 ± 10*
V_T (μl g^{-1})	1.86 ± 0.13	2.6 ± 0.2	1.93 ± 0.13	2.93 ± 0.27
$\dot{V}_E$ (ml g^{-1} min^{-1})	0.28 ± 0.01	0.61 ± 0.05	0.30 ± 0.03	0.81 ± 0.09*

Data presented are mean + S.E.M. * $P < 0.05$ compared with the responses before CIH.

mice within ∼2 h (morning) and ∼8 h (afternoon) after terminating CIH. Control experiments were performed on the same mice wherein ventilation was determined in the morning and afternoon prior to exposing them to 10 days CIH. The results of these experiments are presented in Fig. 6. In the control state prior to CIH, baseline $\dot{V}_E$ was comparable between HET and WT mice when measured in the morning or in the afternoon. However, in CIH-exposed WT mice, $\dot{V}_E$ was significantly higher ∼2 h after terminating CIH (morning) than ventilation monitored after ∼8 h (afternoon). This increase in baseline $\dot{V}_E$ was due to significantly elevated RR ($P < 0.01$). In HET mice, on the other hand, no significant increase in baseline $\dot{V}_E$ was seen ∼2 h after terminating CIH (Fig. 6).

Arterial BP and plasma NA levels. To test the role of HIF-1 in CIH-induced cardiovascular alterations, we measured arterial BP in WT and HET mice. The results are summarized in Fig. 7*A*. Before CIH, systolic, diastolic and mean BPs were comparable in both groups of mice.

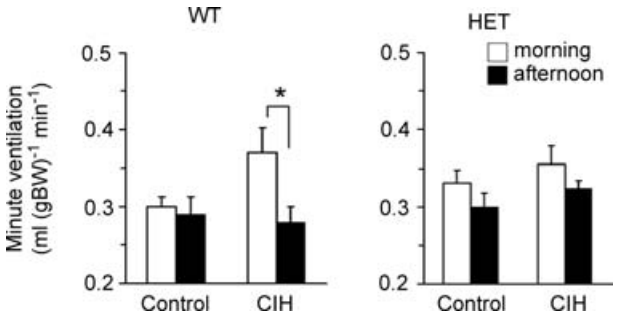

Figure 6. Long-term facilitation (LTF) of breathing
Mean minute ventilation (normalized to body weight (BW)) while breathing 21% O_2 in unanaesthetized *Hif1a*$^{+/+}$ (WT) and *Hif1a*$^{+/-}$ (HET) mice monitored 2 h (morning) and 8 h (afternoon) after completing 10 days of CIH. Increased minute ventilation measured soon after terminating CIH (morning) was considered LTF of breathing. Control, minute ventilation before CIH. Data presented are mean + S.E.M. from eight mice in each group. *$P < 0.05$.

After CIH, BP was significantly elevated in WT but not in HET mice. The increase in BP in WT mice was due to elevations in both systolic and diastolic BPs (Fig. 7*A*). Since CIH increases sympathetic nerve activity (Kara *et al.* 2003), plasma NA levels were measured as an index of sympathetic activation. Basal levels of plasma NA tended to be less in HET than WT mice but the differences were not statistically significant ($P > 0.05$). Following CIH, however, NA levels were significantly elevated in WT but not in HET mice (Fig. 7*B*).

CIH up-regulates HIF-1α protein levels

To test whether CIH increases HIF-1α protein expression, immunoblot assays were performed on cerebral cortex from WT and HET mice exposed either to CIH or to normoxia (control). Cortical tissue was chosen because it yielded adequate protein for performing Western blot assay in individual mice. A representative example of the immunoblot assay and the mean data obtained from densitometric analysis are presented in Fig. 8. Basal HIF-1α expression was significantly lower in HET than WT mice. HIF-1α expression was significantly increased in CIH-exposed WT mice but not in HET mice exposed to CIH. HIF-1β expression was similar in control as well as CIH-exposed WT and HET mice (Fig. 8).

Effect of CIH on ROS levels

Previous studies suggested that CIH increases ROS and that antioxidants prevent cardio-respiratory responses to CIH (Peng & Prabhakar, 2003; Kumar *et al.* 2006). We examined whether CIH increases ROS in mice and, if so, whether HIF-1 contributes to this response. Levels of thio-barbituric acid reactive substances (TBARS) were monitored in cortical tissue samples as a measure of ROS (Ramanathan *et al.* 2005). Basal TBARS were comparable between WT and HET mice (Fig. 9*A*). In CIH-exposed WT mice TBARS were significantly ($P < 0.01$) elevated by

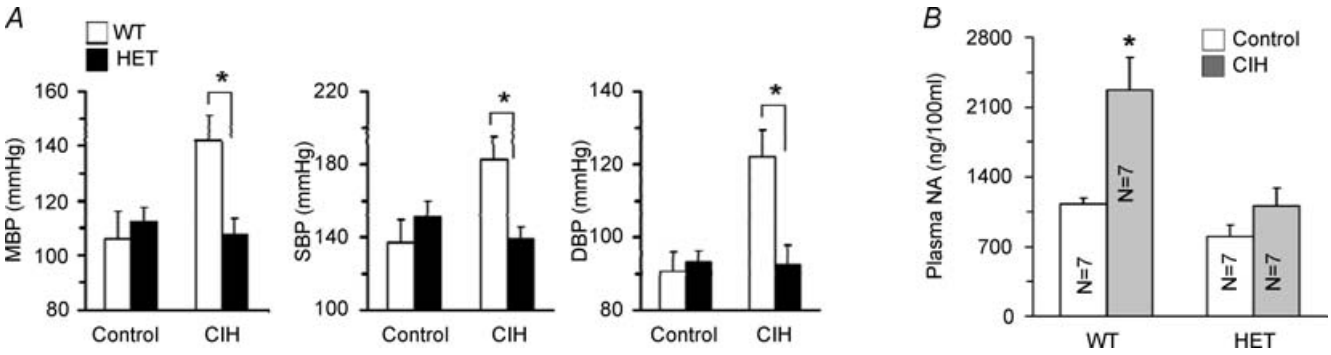

Figure 7. Effect of CIH on blood pressure (BP) and plasma noradrenaline (NA) levels
A, mean BP in *Hif1a*$^{+/+}$ (WT, open bars, $n = 8$) and *Hif1a*$^{+/-}$ (HET, filled bars, $n = 8$) mice, measured before (control) and after 10 days of CIH. MBP, mean blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure (in mmHg). *B,* mean plasma NA in WT and HET mice exposed either to normoxia (Control) or 10 days of CIH. *N*, number of mice. Data presented in *A* and *B* are mean + S.E.M. *$P < 0.05$.

70% and MnTMPyP, a potent scavenger of $O_2^{\bullet -}$, abolished this response (Fig. 9*A* and *B*). MnTMPyP, however, had no effect on basal TBARS. In contrast, TBARS were not increased in CIH-exposed HET mice (Fig. 9*A*). We further examined whether MnTMPyP affects HIF-1α protein expression in WT mice exposed to CIH. As shown in Fig. 9*B*, MnTMPyP completely prevented CIH-evoked up-regulation of HIF-1α in WT mice, whereas it had no effect on HIF-1β expression or on basal HIF-1α expression.

Discussion

A major finding of the present study was that CIH-induced carotid body-mediated cardio-respiratory changes are absent or markedly attenuated in mice with heterozygous deficiency of HIF-1α. The impaired physiological responses to CIH in HET mice were associated with the absence of HIF-1α up-regulation and increased ROS generation.

Consistent with the previous studies of humans (Cistulli & Sullivan, 1994; Narkiewicz *et al.* 1999) and experimental animals (Fletcher *et al.* 1992; Peng & Prabhakar, 2003; Rey *et al.* 2004; Kumar *et al.* 2006), we observed augmented HVR, elevated BP, and plasma NA levels in CIH-exposed WT mice. Although prior to CIH, HVR, BP and plasma NA were comparable between WT and HET mice, CIH was virtually ineffective in affecting these physiological parameters in HET mice. *A priori,* it was possible that the absence of cardio-responses in HET mice might be due to their inability to sense the CIH stimulus. However, this explanation is unlikely because both groups of mice responded with augmented ventilatory response to CO_2 following CIH. The striking absence of HVR in HET mice is not secondary to alterations in metabolic variables and blood gas composition because $\dot{V}_{CO_2}$ and blood gases were comparable in CIH-exposed WT and HET mice. The augmented HVR in CIH-exposed WT mice was also reflected in an increased $\dot{V}_E/\dot{V}_{O_2}$ ratio, a measure of convective requirement, which was unaltered in HET mice. Changes in body temperature can influence HVR (Gautier, 1996). In the present experiments, body temperature was not monitored during HVR. However, it was previously reported that 5 min of 12% O_2 had no significant effect on body temperature in mice (Kline *et al.* 1998). Therefore, it is unlikely that changes in body temperature would explain the absence of augmented HVR in CIH-exposed HET mice. LTF of breathing represents a form plasticity of respiratory motor output that is elicited by repetitive hypoxia (Mitchell & Johnson, 2003). The fact that LTF of breathing was elicited in WT but not HET mice exposed

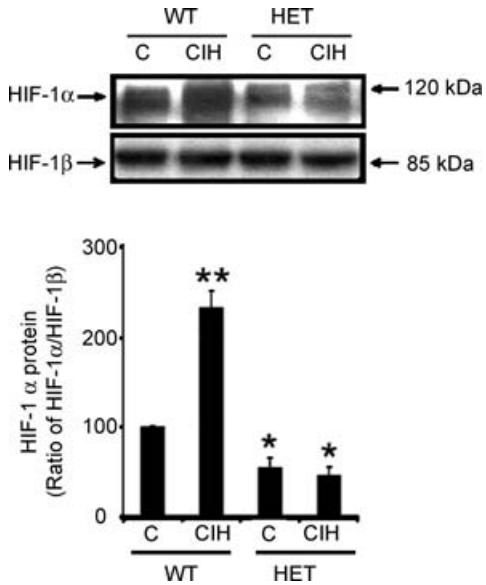

Figure 8. Effect of CIH on HIF-1α protein expression
Top panel, immunoblots of HIF-1α and HIF-1β proteins in cerebral cortex from WT (*Hif-1a*$^{+/+}$) and HET (*Hif-1a*$^{+/-}$) mice exposed to normoxia (C) or CIH. Bottom panel, average data of densitometric analysis of HIF-1α expression relative to HIF-1β. Data are mean + S.E.M. from 5 mice in each group.**$P < 0.01$, *$P < 0.05$.

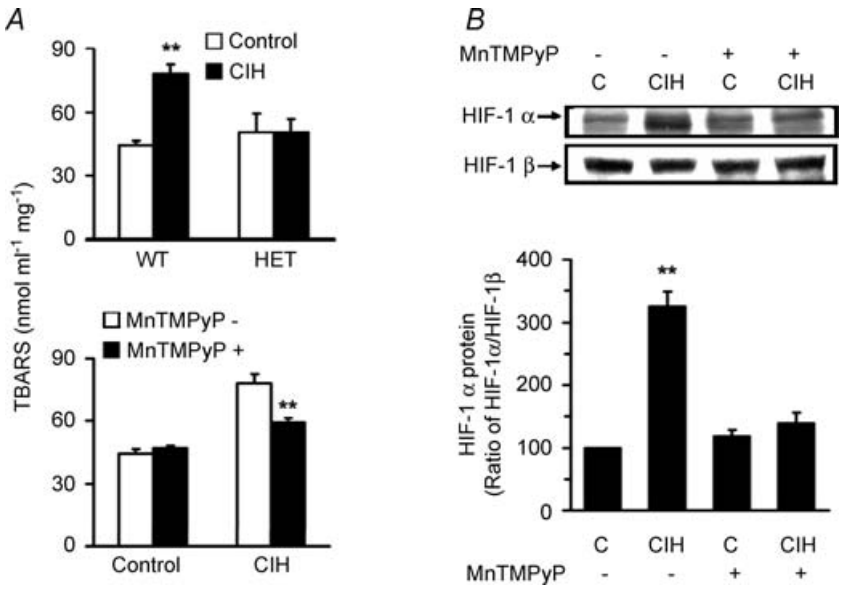

Figure 9. CIH-induced ROS generation and HIF-1α expression
A, analysis of thiobarbituric acid reactive substances (TBARS) as an index of ROS generation. Top panel, mean TBARS in cerebral cortex from WT (*Hif1a*$^{+/+}$) and HET (*Hif1a*$^{+/-}$) mice exposed either to normoxia (Control) or to 10 days of CIH. Bottom panel, effect of MnTMPyP treatment on TBARS in control and CIH-exposed WT mice. Data presented are mean + S.E.M. from 5 mice in each group. *B,* analysis of ROS-induced HIF-1α expression. Top panel, immunoblot assay of HIF-1α and HIF-1β protein expression in cerebral cortex from WT mice, which were exposed to normoxia (C) or 10 days of CIH, either with or without concurrent MnTMPyP administration (5 mg kg^{-1} day^{-1}). Bottom panel, densitometry data (mean + S.E.M.) presented as the HIF-1α/HIF-1β ratio from immunoblot assays of 5 mice in each group.

to CIH suggests that heterozygous deficiency of HIF-1α impairs not only CIH-evoked changes in HVR, BP and plasma NA but also hypoxia-induced plasticity of the respiratory motor output.

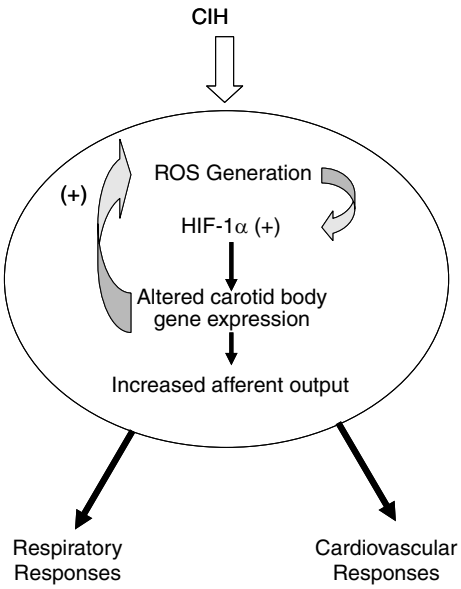

Figure 10. Proposed positive interactions between ROS and HIF1
Feedforward mechanism for ROS generation, HIF-1 activation of gene expression in the carotid body and cardio-respiratory responses to CIH.

Studies in humans (Narkiewicz *et al.* 1999) and experimental animals (Fletcher *et al.* 1992; Peng & Prabhakar, 2003; Rey *et al.* 2004) suggest that the carotid body, which is the primary chemoreceptor for detecting changes in arterial P_{O_2}, mediates CIH-evoked increases in HVR, sympathetic activation and BP. In the present study, carotid body function was assessed by two approaches: first, by recording the ventilatory response to brief hyperoxia, an indirect measure of peripheral chemoreceptor function (Dejour's test), and second, by directly monitoring the sensory activity of 'single' chemoreceptor units from *ex vivo* superfused carotid bodies, wherein the influence from cardiovascular changes on chemoreceptor activity were effectively absent. Both approaches revealed significant augmentation of the carotid body response to hypoxia in CIH-exposed WT mice, a finding that is consistent with previous studies of rats (Peng & Prabhakar, 2004) and cats (Rey *et al.* 2004). The magnitude of the hyperoxia-evoked ventilatory depression (Dejour's test) in control WT mice seen in the present study was less than that reported earlier (Kline *et al.* 2002). However, the current study was performed using only male mice, whereas mice from both genders were used in the earlier study (Kline *et al.* 2002). Therefore, gender differences might account for the lower magnitude of the ventilatory depression by hyperoxia seen in the present study.

We also found a markedly blunted hypoxic sensory response in control HET mice (i.e. prior to CIH exposure). In striking contrast to WT mice, CIH had little effect in augmenting the hypoxic sensory response in HET mice. In addition to its effect on the hypoxic sensory response, CIH has been shown to induce functional plasticity in the rat carotid body, which is manifested as sLTF (Peng *et al.* 2003). Our results demonstrate that CIH was effective in inducing sLTF in WT but not in HET mice. These observations demonstrate that HIF-1, in addition to enhancing the hypoxic sensory response, also plays a critical role in inducing functional plasticity of the carotid body by CIH, and suggest that the absence of CIH-evoked cardio-respiratory responses in HET mice is primarily due to impaired carotid body function resulting from HIF-1α deficiency.

The augmented carotid body-mediated cardio-respiratory responses to CIH were associated with up-regulation of HIF-1α protein expression in WT mice. The up-regulation of HIF-1α by CIH is similar to that reported with chronic sustained hypoxia in rats. HIF-1α expression progressively decreases with continued exposure to sustained hypoxia (Chavez *et al.* 2000). Whether a similar decrease in HIF-1α expression also occurs with continued exposure to CIH, however, remains to be investigated. Nonetheless, the present observations taken together with published data (Chavez *et al.* 2000) indicate that both chronic sustained and CIH up-regulate HIF-1α expression in intact animals.

As expected, basal expression of HIF-1α protein was reduced by $\sim$50% in mice with heterozygous deficiency of HIF-1α. We anticipated increased HIF-1α levels in CIH-exposed HET mice, albeit of a lower magnitude compared with WT mice. Contrary to our expectation, we observed no increased HIF-1α expression in CIH-exposed HET mice. These surprising results suggest the existence of a HIF-1-dependent feed-forward mechanism that induces and/or sustains increased HIF-1α expression in CIH-exposed mice, as discussed in greater detail below. Regardless of the underlying mechanism, these results demonstrate that the complete absence of CIH-evoked physiological responses in HET mice is associated with a total failure to induce HIF-1α expression, thus providing a basis for the dramatic phenotype associated with heterozygous deficiency of HIF-1α.

Recent studies reported that CIH increases ROS in the carotid body (Peng *et al.* 2003), adrenal medulla (Kumar *et al.* 2006), and in the central nervous system (Ramanathan *et al.* 2005). These studies have implicated increased ROS as an important signal in eliciting cardio-respiratory responses to CIH (Peng & Prabhakar, 2003, 2004; Kumar *et al.* 2006). However, which ROS species (O_2^-, H_2O_2, or OH^-) play a role in CIH-induced cardio-respiratory responses and the molecular mechanisms underlying their increased generation during CIH are not known. The following results from the present study indicate that HIF-1 plays role in CIH-induced ROS generation and that there is a positive interaction between ROS and HIF-1. First, ROS levels were elevated in WT but not in HET mice exposed to CIH as evidenced by elevated TBARS in the former but not in the latter. Second, antioxidant treatment prevented both increased generation of ROS and increased HIF-1α expression in CIH-exposed WT mice. We hypothesize that CIH may initially trigger ROS generation, possibly involving inhibition of mitochondrial electron transport chain activity as reported previously (Peng *et al.* 2003; Yuan *et al.* 2004). The increased ROS levels up-regulate HIF-1α expression, as has been described in other experimental contexts (Kietzmann & Gorlach, 2005; Guzy *et al.* 2005). Once HIF-1 is activated, it may function to maintain increased ROS levels. The proposed positive interactions between ROS and HIF-1 are schematically shown in Fig. 10. Verification of this model and delineation of the underlying molecular mechanisms will require further studies. Although HIF-1 controls the expression of hundreds of genes (Manalo *et al.* 2005) that may contribute to the observed phenotype, the data from this study suggest that HIF-1 plays a previously unrecognized, essential role in ROS generation and cardio-respiratory responses to CIH.

References

Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM & Sharp FR (2000). Role of hypoxia-inducible factor−1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann Neurol* **48**, 285–296.

Buelke-Sam J, Holson JF, Bazare JJ & Young JF (1978). Comparative stability of physiological parameters during sustained anesthesia in rats. *Laboratory Anim Sci* **28**, 157–162.

Chavez JC, Agani F, Pichiule P & LaManna JC (2000). Expression of hypoxia-inducible factor−1α in the brain of rats during chronic hypoxia. *J Appl Physiol* **89**, 1937–1942.

Cistulli PA & Sullivan CE (1994). Pathophysiology of sleep apnea. In *Sleep and Breathing*, ed. Saunders NA & Sullivan CE, pp. 405–448. Dekker, New York.

Dejours P (1962). Chemoreceptors in breathing. *Physiol Rev* **42**, 335–358.

Dyugovskaya L, Lavie P & Lavie L (2002). Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* **165**, 934–939.

Fletcher EC, Lesske J, Behm R, Miller CC III, Stauss H & Unger T (1992). Carotid chemoreceptors, systemic blood pressure, and chronic episodic hypoxia mimicking sleep apnea. *J Appl Physiol* **72**, 1978–1984.

Frappell P, Lanthier C, Baudinette RV & Mortola JP (1992). Metabolism and ventilation in acute hypoxia: a comparative analysis in small mammalian species. *Am J Physiol* **262**, R1040–R1046.

Gautier H (1996). Interactions among metabolic rate, hypoxia, and control of breathing. *J Appl Physiol* **81**, 521–527.

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U & Schumacker PT (2005). Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metab* **1**, 401–408.

Hirota K & Semenza GL (2006). Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit Rev Hematol Oncol* **59**, 15–26.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY & Semenza GL (1998). Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. *Genes Dev* **12**, 149–162.

Kara T, Narkiewicz K & Somers VK (2003). Chemoreflexes – physiology and clinical implications. *Acta Physiol Scand* **177**, 377–384.

Kietzmann T & Gorlach A (2005). Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Semin Cell Dev Biol* **16**, 474–486.

Kim DK, Natarajan N, Prabhakar NR & Kumar GK (2004). Facilitation of dopamine and acetylcholine release by intermittent hypoxia in PC12 cells: involvement of calcium and reactive oxygen species. *J Appl Physio* **96**, 1206–1215.

Kline DD, Peng YJ, Manalo DJ, Semenza GL & Prabhakar NR (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1α. *Proc Natl Acad Sci U S A* **99**, 821–826.

Kline DD, Yang T, Huang PL & Prabhakar NR (1998). Altered respiratory responses to hypoxia in mutant mice deficient in neuronal nitric oxide synthase. *J Physiol* **511**, 273–287.

Kumar GK, Rai V, Sharma SD, Ramakrishnan DP, Peng YJ, Souvannakitti D & Prabhakar NR (2006). Chronic intermittent hypoxia induces hypoxia-evoked catecholamine efflux in adult rat adrenal medulla via oxidative stress. *J Physiol* **575**, 229–239.

Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG & Semenza GL (2005). Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* **105**, 659–669.

Mitchell GS & Johnson SM (2003). Neuroplasticity in respiratory motor control. *J Appl Physiol* **94**, 358–374.

Narkiewicz K, van de Borne PJ, Pesek CA, Dyken ME, Montano N & Somers VK (1999). Selective potentiation of peripheral chemoreflex sensitivity in obstructive sleep apnea. *Circulation* **99**, 1183–1189.

Peng YJ, Overholt JL, Kline DD, Kumar GK & Prabhakar NR (2003). Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. *Proc Natl Acad Sci U S A* **100**, 10073–10078.

Peng YJ & Prabhakar NR (2003). Reactive oxygen species in the plasticity of respiratory behavior elicited by chronic intermittent hypoxia. *J Appl Physiol* **94**, 2342–2349.

Peng YJ & Prabhakar NR (2004). Effect of two paradigms of chronic intermittent hypoxia on carotid body sensory activity. *J Appl Physiol* **96**, 1236–1242.

Ramanathan L, Gozal D & Siegel JM (2005). Antioxidant responses to chronic hypoxia in the rat cerebellum and pons. *J Neurochem* **93**, 47–52.

Rey S, Del Rio R, Alcayaga J & Iturriaga R (2004). Chronic intermittent hypoxia enhances cat chemosensory and ventilatory responses to hypoxia. *J Physiol* **560**, 577–586.

Wang GL, Jiang BH, Rue EA & Semenza GL (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci U S A* **92**, 5510–5514.

Wenger RH, Stiehl DP & Camenisch G (2005). Integration of oxygen signaling at the consensus HRE. *Sci STKE* **306**, re12.

Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Weiner CM, Sylvester JT & Semenza GL (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *J Clin Invest* **103**, 691–696.

Yuan G, Adhikary G, McCormick AA, Holcroft JJ, Kumar GK & Prabhakar NR (2004). Role of oxidative stress in intermittent hypoxia-induced immediate early gene activation in rat PC12 cells. *J Physiol* **557**, 773–783.

Yuan G, Nanduri J, Bhasker CR, Semenza GL & Prabhakar NR (2005). Ca^{2+}/calmodulin kinase-dependent activation of hypoxia inducible factor 1 transcriptional activity in cells subjected to intermittent hypoxia. *J Biol Chem* **280**, 4321–4328.

Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW & Semenza GL (2000). Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* **88**, 2606–2618.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL & Simons JW (1999). Overexpression of hypoxia-inducible factor 1 alpha in common human cancers and their metastases. *Cancer Res* **59**, 5830–5835.

Acknowledgements

This work is supported by grants from the National Institutes of Health HL-25830.